Supporting Information

Discovery, Synthesis, and Structure Activity Relationship of a Series of *N*-Aryl-bicyclo[2.2.1]heptane-2-carboxamides: Characterization of ML213 as a Novel KCNQ2 and KCNQ4 Potassium Channel Opener

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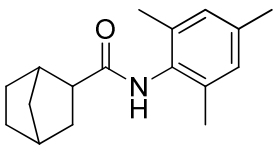
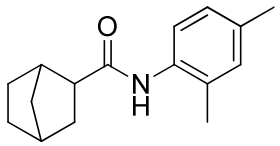
Experimental Section

General. All NMR spectra were recorded on a 400 MHz AMX Bruker NMR spectrometer. ^{1}H chemical shifts are reported in δ values in ppm downfield with the deuterated solvent as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constant (Hz). Low resolution mass spectra were obtained on an Agilent 1200 series 6130 mass spectrometer with electrospray ionization. High resolution mass spectra were recorded on a Waters Q-TOF API-US plus Acquity system with electrospray ionization. Analytical thin layer chromatography was performed on EM Reagent 0.25 mm silica gel 60-F plates. Analytical HPLC was performed on an Agilent 1200 series with UV detection at 214 nm and 254 nm along with ELSD detection. LC/MS: Method 1 = (J-Sphere80-C18, 3.0 x 50 mm, 4.1 min gradient, 5%[0.05%TFA/CH_3CN]:95%[0.05%TFA/H_2O] to 100%[0.05%TFA/CH_3CN]; Method 2 = (Phenomenex-C18, 2.1 X 30 mm, 2 min gradient, 7%[0.1%TFA/CH_3CN]:93%[0.1%TFA/H_2O] to 100%[0.1%TFA/CH_3CN]; Method 3 = (Phenomenex-C18, 2.1 X 30 mm, 1 min gradient, 7%[0.1%TFA/CH_3CN]:93%[0.1%TFA/H_2O] to 95%[0.1%TFA/CH_3CN]. Preparative purification was performed on a custom HP1100 purification system (reference 16) with collection triggered by mass detection. Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification.



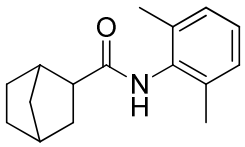
***N*-Mesitylbicyclo[2.2.1]heptane-2-carboxamide (ML213, 1).**

General Procedure: To a solution of 2-norbornanecarbonylchloride (1.0 eq, 250 mg, 1.6 mmol) in DMF (1 mL) was added 2,4,6-trimethylaniline (213 mg, 1.6 mmol) and triethylamine (440 μL, 3.2 mmol). After stirring 24 h, the reaction mixture was filtered and the supernatant purified by mass directed chromatography to provide **ML213** (124.3 mg, 0.5 mmol, 30%) as a white crystalline solid. LCMS: R_T = 0.766 min., *m/z* = 258.4 $[M + H]^+$, >98% at 215 and 254 nm. ^1H NMR (400 MHz, $CDCl_3$) δ 6.89 (s, 2H), 6.61 (bs, 1H), 2.90-2.84 (m, 1H), 2.58 (d, *J* = 5.2 Hz, 1H), 2.37-2.31 (m, 1H), 2.26 (s, 3H), 2.19 (s, 3H), 2.18 (s, 3H), 1.79-1.73 (m, 1H), 1.67-1.21 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3, 136.6, 135.1, 131.5, 128.8, 47.4, 41.3, 36.9, 34.3, 31.5, 29.3, 24.4, 20.8, 18.5, 18.3. HRMS, calc'd for $C_{17}H_{24}NO$ $[M + H]^+$, 258.1858; found 258.1860.



***N*-(2,4-Dimethylphenyl)bicyclo[2.2.1]heptane-2-carboxamide (2).**

LCMS: R_T = 0.781 min., *m/z* =244.3 $[M + H]^+$, >98% @ 215 and 254 nm. ^1H NMR (400 MHz, $CDCl_3$) δ 7.70 (dd, *J* = 8.0, 7.6 Hz, 1H), 7.02 (d, *J* = 8.4 Hz, 1H), 6.87 (bs, 1H), 2.84 (t, *J* = 5.2 Hz, 1H), 2.54 (bs, 1H), 2.29 (s, 3H), 2.23 (s, 3H), 1.79 (ddd, *J* = 7.2, 4.8, 2.0 Hz, 1H), 1.69-1.20 (m, 8 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.1, 134.4, 133.3, 130.9, 127.2, 123.0, 48.0, 41.3, 40.6, 36.9, 34.3, 31.4, 29.1, 24.4, 20.8, 17.7. HRMS, calc'd for $C_{16}H_{22}NO$ $[M + H]^+$, 244.1701; found 244.1703.



***N*-(2,6-Dimethylphenyl)bicyclo[2.2.1]heptane-2-carboxamide (3).**

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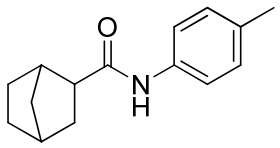
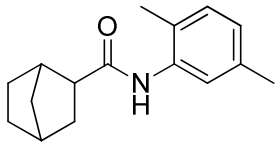


***N*-(*p*-Tolyl)bicyclo[2.2.1]heptane-2-carboxamide (4).**

LCMS: R_T = 0.778 min., *m/z* = 230.3 $[M + H]^+$, >98% @ 215 and 254 nm. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (d, *J* = 8.4 Hz, 2H), 7.12 (d, *J* = 8.4 Hz, 2H), 2.81-2.75 (m, 1H), 2.52 (d, *J* = 4.0 Hz, 1H) 2.31 (s, 3H), 2.02-1.96 (m, 1H), 1.79 (ddd, *J* = 7.2, 4.8, 2.0 Hz, 1H), 1.72-1.18 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 135.6, 133.4, 129.4, 119.6, 48.1, 40.6, 37.0, 34.1, 31.3, 29.1, 24.3, 20.7. HRMS, calc'd for $C_{15}H_{20}NO$ $[M + H]^+$, 230.1545; found 230.1544.



N-(2,5-Dimethylphenyl)bicyclo[2.2.1]heptane-2-carboxamide (5).

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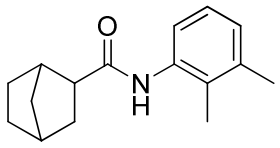
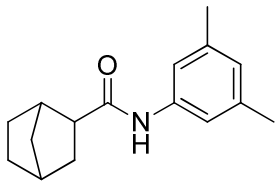


***N*-(2,3-Dimethylphenyl)bicyclo[2.2.1]heptane-2-carboxamide (6).**

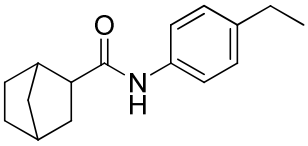
LCMS: R_T = 0.709 min., *m/z* = 244.3 $[M + H]^+$, >98% at 215 and 254 nm. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (dd, *J* = 7.6, 7.6 Hz, 1H), 7.12-7.08 (m, 1H), 7.00 (d, *J* = 7.2 Hz, 1H), 6.95 (bs, 1H), 2.86 (t, *J* = 6.0, 4.8 Hz, 1H), 2.55 (d, *J* = 3.2 Hz, 1H), 2.31 (s, 3H), 2.16 (s, 3H), 1.83 (ddd, *J* = 7.2, 4.8, 2.4 Hz, 1H), 1.75-1.21 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3, 135.6, 135.5,

126.8, 125.8. 121.8, 47.9, 40.6, 36.9 34.3, 31.4, 29.1, 24.4, 20.5, 13.6. HRMS, calc'd for $C_{16}H_{22}NO$ $[M + H]^+$, 244.1701; found 244.1701.



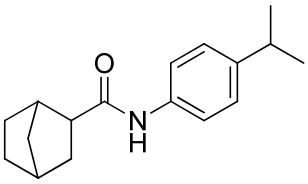
***N*-(3,5-Dimethylphenyl)bicyclo[2.2.1]heptane-2-carboxamide (7).**

LCMS: R_T = 0.805 min., *m/z* = 244.3 $[M + H]^+$, >98% @ 215 and 254 nm.



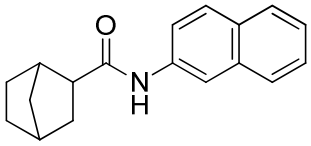
***N*-(4-Ethylphenyl)bicyclo[2.2.1]heptane-2-carboxamide (8).**

LCMS: R_T = 0.807 min., *m/z* = 244.3 $[M + H]^+$, >98% @ 215 and 254 nm.



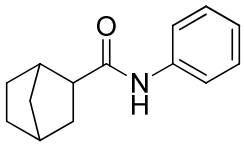
***N*-(4-Isopropylphenyl)bicyclo[2.2.1]heptane-2-carboxamide (9).**

LCMS: R_T = 0.852 min., *m/z* = 258.4 $[M + H]^+$, >98% at 215 and 254 nm. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (d, *J* = 7.2 Hz, 2H), 7.17 (dd, *J* = 8.4, 2.4 Hz, 2H), 2.91-2.84 (m, 1H), 2.81-2.76 (m, 1H), 2.52 (d, *J* = 4.4 Hz, 1H), 2.34-2.24 (m, 1H), 1.80 (ddd, *J* = 7.2, 4.8, 2.4 Hz, 1H), 1.71-1.64 (m, 1H), 1.56-1.33 (m, 7H), 1.24 (s, 3H), 1.23 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 144.6, 135.9, 126.8, 119.7, 48.1, 40.6, 37.0, 34.1, 33.5, 31.3, 29.1, 24.3, 24.0. HRMS, calc'd for $C_{17}H_{24}NO$ $[M + H]^+$, 258.1858 found; 258.1859.



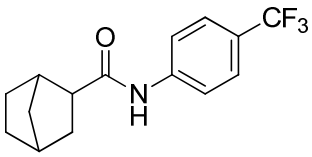
***N*-(Naphthalen-2-yl)bicyclo[2.2.1]heptane-2-carboxamide (10).**

LCMS: R_T = 0.780 min., *m/z* = 266.4 $[M + H]^+$, >97% @ 215 and 254 nm. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (d, *J* = 6.0 Hz, 1H), 7.78 (d, *J* = 8.0 Hz, 3H), 7.47-7.37 (m, 3H), 2.88-2.82 (m, 1H), 2.57 (s, 1H), 2.37-2.31 (m, 1H), 2.06-2.01 (m, 1H), 1.88 (ddd, *J* = 7.2, 4.8, 2.4 Hz, 1H), 1.76-1.69 (m, 1H), 1.52-1.38 (m, 5H), 1.32-1.23 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6, 135.6, 133.9, 130.4, 128.6, 127.5, 127.4, 126.4, 124.8, 119.7, 116.2, 48.3, 40.7, 37.0, 34.2, 31.4, 29.1, 24.4. HRMS, calc'd for $C_{18}H_{20}NO$ $[M + H]^+$, 266.1545; found 266.1544



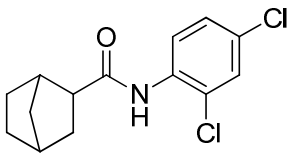
***N*-Phenylbicyclo[2.2.1]heptane-2-carboxamide (11).**

LCMS: R_T = 0.742 min., *m/z* = 216.3 $[M + H]^+$, >98% @ 215 and 254 nm.



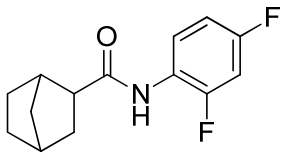
***N*-(4-(Trifluoromethyl)phenyl)bicyclo[2.2.1]heptane-2-carboxamide (12).**

LCMS: R_T = 0.827 min., *m/z* = 284.3 $[M + H]^+$, >98% @ 215 and 254 nm.



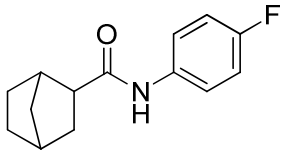
***N*-(2,4-Dichlorophenyl)bicyclo[2.2.1]heptane-2-carboxamide (13).**

LCMS: R_T = 0.845 min., *m/z* = 285.2 $[M + H]^+$, >98% @ 215 and 254 nm.



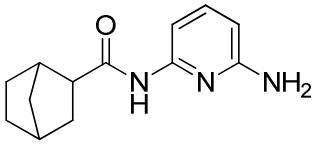
***N*-(2,4-Difluorophenyl)bicyclo[2.2.1]heptane-2-carboxamide (14).**

LCMS: R_T = 0.757 min., *m/z* = 252.3 $[M + H]^+$, >98% @ 215 and 254 nm.



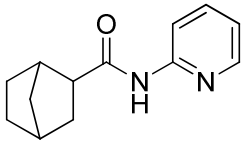
***N*-(4-Fluorophenyl)bicyclo[2.2.1]heptane-2-carboxamide (15).**

LCMS: R_T = 0.753 min., *m/z* = 234.3 $[M + H]^+$, >98% @ 215 and 254 nm.



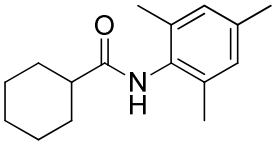
***N*-(6-Aminopyridin-2-yl)bicyclo[2.2.1]heptane-2-carboxamide (16).**

LCMS: R_T = 0.836 min., *m/z* = 258.4 $[M + H]^+$, >98% @ 215.



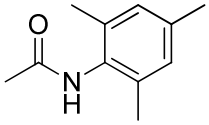
***N*-(Pyridin-2-yl)bicyclo[2.2.1]heptane-2-carboxamide (17).**

LCMS: R_T = 0.649 min., *m/z* = 217.3 $[M + H]^+$, >98% @ 215 and 254 nm.



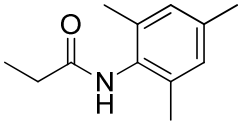
***N*-mesitylcyclohexanecarboxamide (18).**

LCMS: R_T = 0.773 min., *m/z* = 246.4 $[M + H]^+$, >98% @ 215 and 254 nm.



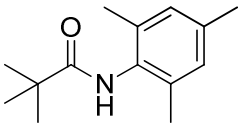
***N*-mesitylacetamide (19).**

LCMS: R_T = 0.618 min., *m/z* = 178.2 $[M + H]^+$, >98% @ 215 and 254 nm.



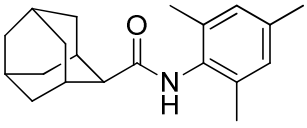
***N*-mesitylpropionamide (20).**

LCMS: R_T = 0.651 min., *m/z* = 192.3 $[M + H]^+$, >98% @ 215 and 254 nm.



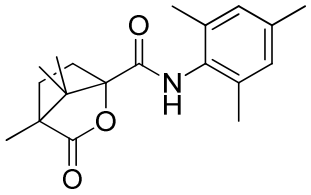
***N*-mesitylpivalamide (21).**

LCMS: R_T = 0.749 min., *m/z* = 220.3 $[M + H]^+$, >97% @ 215.



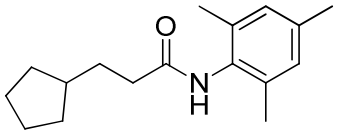
***N*-mesityladamantane-2-carboxamide (22).**

LCMS: R_T = 0.843 min., *m/z* = 298.4 $[M + H]^+$, >98% @ 215 and 254 nm.



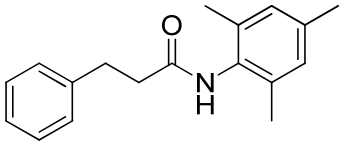
***N*-Mesityl-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carboxamide (23).**

LCMS: R_T = 0.771 min., *m/z* = 316.4 $[M + H]^+$, >98% @ 215 and 254 nm.



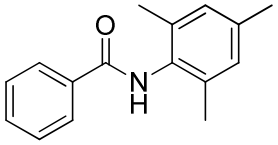
3-Cyclopentyl-*N*-mesitylpropanamide (24).

LCMS: R_T = 0.810 min., *m/z* = 260.4 $[M + H]^+$, >98% @ 215 and 254 nm.



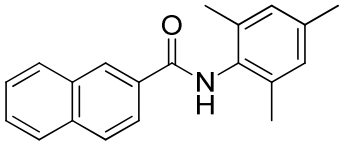
***N*-Mesityl-3-phenylpropanamide (25).**

LCMS: R_T = 0.773 min., *m/z* = 268.4 $[M + H]^+$, >98% @ 215 and 254 nm.



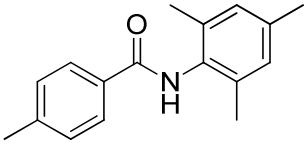
***N*-Mesitylbenzamide (26).**

LCMS: R_T = 0.739 min., *m/z* = 240.2 $[M + H]^+$, >98% @ 215 and 254 nm.



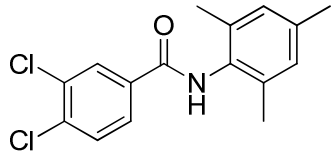
***N*-Mesityl-2-naphthamide (27).**

LCMS: R_T = 0.803 min., *m/z* = 290.4 $[M + H]^+$, >95% @ 215 and 254 nm.



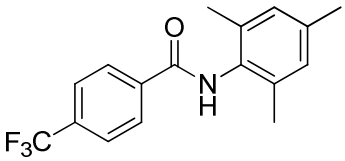
***N*-Mesityl-4-methylbenzamide (28).**

LCMS: R_T = 0.770 min., *m/z* = 254.3 $[M + H]^+$, >98% @ 215 and 254 nm.



3,4-Dichloro-*N*-mesitylbenzamide (29).

LCMS: R_T = 0.826 min., *m/z* = 309.2 $[M + H]^+$, >98% @ 215 and 254 nm.



***N*-Mesityl-4-(trifluoromethyl)benzamide (30).**

LCMS: R_T = 0.808 min., *m/z* = 308.3 $[M + H]^+$, >98% @ 215 and 254 nm.

In vitro PK Analysis:

Microsomal stability: The metabolic stability of each compound was investigated in rat hepatic microsomes (BD Biosciences, Billerica, MA) using substrate depletion methodology (% parent compound remaining). In separate 96-well plates for each time point, a mixture of 0.1M potassium phosphate-buffered (pH 7.4), 1μM test compound, 0.5 mg/mL microsomes, and 1mM NADPH (t=3, 7, 15, 25, or 45min) or buffer (t=0) were continually incubated at 37°C under ambient oxygenation. At the respective time, each plate's reaction was precipitated by the addition of 2 volumes of ice-cold acetonitrile containing glyburide as an internal standard (50 ng/mL). The plates were centrifuged at 3000 rpm (4°C) for 10 min. The resulting supernatants were transferred and diluted 1:1 (supernatant: water) into new 96-well plates in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate. The in vitro half-life ($t_{1/2}$, min, Eq. 1), intrinsic clearance (*CLint*, mL/min/kg, Eq. 2) and subsequent predicted hepatic clearance (*CLhep*, mL/min/kg, Eq. 3) was determined employing the following equations:

1) $t_{1/2}$ = Ln(2) / k ; where k represents the slope from linear regression analysis (% test compound remaining)

2) *CLint* = (0.693 / $t_{1/2}$) (rxn volume / mg of microsomes) (45 mg microsomes / gram of liver) (20[a] gm of liver / kg body weight); [a]scale-up factors of 20 (human) and 45 (rat)

3) $$CLhep = \frac{Q \cdot CL\,\mathrm{int}}{Q + CL\,\mathrm{int}}$$

Plasma Protein Binding. The protein binding of each compound was determined in rat plasma via equilibrium dialysis employing Single-Use RED Plates with inserts (ThermoFisher Scientific, Rochester, NY). Plasma (220 µL) was added to the 96 well plate containing test compound (5 µL) and mixed thoroughly. Subsequently, 200 µL of the plasma-compound mixture was transferred to the *cis* chamber (red) of the RED plate, with an accompanying 350 µL of phosphate buffer (25 mM, pH 7.4) in the *trans* chamber. The RED plate was sealed and incubated for 4 hours at 37°C with shaking. At completion, 50 µL aliquots from each chamber were diluted 1:1 (50 µL) with either plasma (*cis*) or buffer (*trans*) and transferred to a new 96 well plate, at which time ice-cold acetonitrile (2 volumes) was added to extract the matrices. The plate was centrifuged (3000 rpm, 10 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate.

Microsomal stability and plasma protein binding samples were analyzed on a Thermo Electron TSQ Quantum Ultra triple quad detector via electrospray ionization (ESI) with two Themo Electron Accella pumps (San Jose, CA), and a Leap Technologies CTC PAL autosampler. Analytes were separated by gradient elution on a dual column system with two Waters Acquity BEH C18, 2.1x50mm, 1.7µm columns (Milford, MA) heated at 50°C. HPLC mobile phase A was 95:5:0.1 Water:Acetonitrile:Formic Acid, while mobile phase B was 95:5:0.1 Acetonitrile:Water:Formic Acid. Pump 1 runs the gradient: 95:5 (A:B) at 800µL/min hold 0 to 0.5min, linear ramp to 5:95 (A:B) 0.5 to 1.0min, 5:95 (A:B) hold 1.0 to 1.9min, return to 95:5 (A:B) at 1.9 min. While pump 1 runs the gradient

method, pump 2 equilibrates the other column isocratically with 95:5 (A:B). The total run time is 2.0 minutes per injection. All compounds are optimized using Thermo Electron's QuickQuan software.

Cytochrome P450 inhibition: A four-in-one, 96-well plate assay for determining IC_{50} values against human P450s 1A2, 2C9, 2D6 and 3A4 was developed based on previous reports (1,2). Human liver microsomes (final concentration of 0.1 mg/mL) and a substrate mixture containing the P450 probe substrates phenacetin (10μM), diclofenac (5μM), dextromethorphan (5μM) and midazolam (2μM) were added to a potassium phosphate buffered solution (0.1M, pH 7.4) and warmed to 37°C. The reaction mixture was divided evenly into the 96-well plate and various dilutions of each inhibitor/compound of interest (in duplicate) were then added to this reaction mixture such that the final concentration of each compound ranged from 100nM to 30μM. This mixture was allowed to pre-incubate for 15 minutes while shaking at 37°C. Buffer or NADPH (1mM) was added and the reaction mixture was incubated for an additional 8 minutes at 37°C prior to quenching with 2 volumes of ice-cold acetonitrile containing 50ng/mL of carbamazepine as internal standard. The plates were centrifuged at 4000 rpm (4°C) for 10 minutes and the supernatant was removed and diluted with water (1:4, v/v) in preparation for LC/MS/MS analysis. The IC_{50} values for each compound were obtained for the individual P450 enzymes by quantitating the inhibition of metabolite formation for each probe; acetaminophen (1A2), 4-hydroxydiclofenac (2C9), dextrorphan tartrate (2D6) and 1-hydroxymidazolam (3A4). Miconazole was included as a positive control broad spectrum P450 inhibiton (REF). For discrete 2C19 inhibition experiments,

a similar assay design was employed with the following exceptions: the probe substrate was S-mephenytoin (40μM), the NADPH incubation with the reaction mixture went for 30 minutes, the supernatant was reconstituted 1:1 with water for analysis, and the metabolite used for quantitation was 4-hydroxymephenytoin.

Cytochrome P450 inhibition samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 2.1 x 50 mm, 3 μm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 10% B after a 0.2 min hold and was linearly increased to 90% B over 1.3 min; returned to 10% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500°C and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciex Analyst 1.4.2 software.

In vivo PK:

IP PBL: Compound was formulated as 10% Tween 80 in sterile water at the concentration of 3 mg/mL and administered i.p. to male Sprague-Dawley rats weighing 250 g (Harlan, Indianapolis, IN) at the dose of 10 mg/kg. The rat blood (cardiac puncture) and brain were collected at 1 hour. Animals were euthanized and decapitated, and the brains were removed, thoroughly washed in cold phosphate-buffered saline, and immediately frozen on dry ice. Plasma was separated by centrifugation and stored at -80°C until analysis. On the day of analysis, frozen whole-rat brains were weighed and diluted with 1:3 (w/w) parts of 70:30 isopropanol:water. The mixture was then subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products) followed by centrifugation. The sample extraction of plasma (20 µl) and brain homogenate (20 µl) was performed by a method based on protein precipitation using 120µL acetonitrile containing an internal standard (50 ng/mL carbamazepine). Extracts were centrifuged at 4000 x g for 5 min. The supernatants of plasma and brain homogenate extracts were then diluted 1:1 with water.

Liquid chromatography/mass spectrometry analysis: In vivo samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 3.0 x 30 mm, 3 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 10% B after a 0.2 min hold and was linearly increased to 90% B over 0.8 min; held at 90% B for 0.5 min and returned to 10% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at

500°C and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage) (see Table 1). All data were analyzed using AB Sciex Analyst 1.5.1 software.

In Vitro Pharmacology

Cell culture

Stable lines expressing KCNQ2 were generated by standard protocols using pcDNA3.1(+) and cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 medium(DMEM/F12) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Gemini, Calabasas, CA), 2 mM L-Glutamine (Invitrogen, Carlsbad, CA) and 500 μg/mL G418 (Invitrogen, Carlsbad, CA). Cells have been maintained and passaged when reaching 80% confluency.

Thallium-based fluorescence assay

Activity of potassium channels was monitored by the influx of a surrogate ion for potassium, thallium (Tl+). Thallium influx was detected through the use of a thallium-sensitive fluorescent dye, FluxOR^{TM} (Invitrogen, Carlsbad, CA). Cells were plated using a Multidrop (Thermo scientific, Hudson, New Hampshire) at 6,500 cells/well into BD Biocoat poly-D-Lysine coated 384-well plates and incubated overnight (16-20 hr) at 37°C and 5% CO2. The thallium-based fluorescence assay protocol was adapted from the manufacturer's recommended protocol. Briefly, medium was removed; cells were loaded with 1x FluxOR dye solution, 25 μl/well, for 90 min at room temperature in the dark; the 1x FluxOR solution was replaced by assay buffer (Hanks Balanced Salt Solution containing 5.8 mM potassium; Catalog # 14065, Invitrogen), 20 μl/well; test compounds at final concentration 10 μM from a 5 mM stock supplied by the NIH Molecular Libraries Small Molecule Repository (BioFocus DPI, San Francisco, CA) or controls in assay buffer were then added to cells, 4 μl /well; 20 minutes later, cell plates were loaded to Hamamatsu FDSS 6000 kinetic imaging plate reader (Hamamatsu Photonics, Hamamatsu city, Japan); after establishing fluorescence baseline by 1 Hz scanning for 10 seconds, the KCNQ2 channels were activated by addition of 6 μl/well stimulus buffer containing 12.5 mM K_2SO4 and 12.5 mM Tl_2SO4 to the assay buffer giving a final potassium concentration of 15.8 mM and final

Tl+ concentration of 5 mM; fluorescence measurement was continued at 1 Hz for another 110 seconds. To evaluate the robustness of the HTS thallium-based fluorescence assays, ZTZ240 at 10 μM was applied as positive activator control, while assay buffer was employed as negative control. Both the positive and negative controls are prepared with 0.2% (v/v) DMSO, corresponding to the test concentration 10 μM. The fluorescence ratio, F(max-min)/F0 (ΔF/F0), was calculated for each well using the entire 120 second detection window and then normalized to the positive and negative control wells. Hit selection was based on the B scores of test compounds calculated from the fluorescence ratios. If the B score of the test compound was more than 3 times the standard deviation (SD) of the B scores of ratios of the library compounds, and the B score of initial fluorescence intensity is within 2 times the standard deviation of the B scores of the library compounds, the compound is designated as an activator of KCNQ2 channels. Otherwise, it is designated as inactive.

Automated electrophysiology assay

KCNQ2 activity was examined in an electrophysiological assay using the population patch clamp mode on the Ionworks Quattro (MDC, Sunnyvale, CA), an automated patch clamp instrument. The CHO cells stably expressing KCNQ2 channels were freshly dislodged from flasks and dispensed into a 384-well population patch clamp (PPC) plate. The cell plating density was 7,000 cells/well suspended in the extracellular solution, composed of (in mM): 137 NaCl, 4 KCl, 1 MgCl2, 1.8 CaCl2, 10 HEPES, and 10 glucose, pH 7.4 adjusted with NaOH.

After dispensing, seal resistance of cells was measured for each well and cells were perforated by incubation with 50μg/ml amphotericin B (Sigma, St. Louis, MO), which was dissolved in the internal solution composed of (in mM): 40 KCl, 100 K-Gluconate, 1 MgCl2, 5 HEPES, 2 CaCl2, pH 7.2 adjusted with KOH. Activity of KCNQ2 was then measured with the recording protocol

as followings. Leak currents were linear subtracted extrapolating the current elicited by a 100-ms step to -100 mV from a holding potential of -90 mV. During the voltage pulse protocol, cells were held at -90 mV, followed by by 2,000 ms depolarizing step from -90 mV to -10 mV, and then back to -90mV for 2000 ms. The currents were measured at the end of the depolarization pulse before and after the application of compounds for 3 min. Only cells with a current amplitude more than 100 pA at -10 mV and a seal resistance >30 MΩ were included in the data analysis.

Compound effects were assessed by the percentage changes in the KCNQ2 steady state currents, which were calculated by dividing the difference between pre- and post-compound KCNQ2 currents by the respective pre-compound currents in the same well. When constructing conductance-voltage curves, conductance values were calculated by dividing the steady state outward currents measured during the voltage steps by the driving force (step voltage minus the calculated potassium reversal potential).

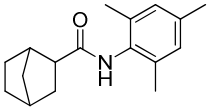
The KCNQ2 protocol was also used for KCNQ2/KCNQ3 recording. But for KCNQ1 and KCNQ4, the cells were depolarized to +40mV from the holding potential -70 mV. Currents were measured at the step current at +40 mV. And for KCNQ1/KCNE1, cells were stimulated by 3,000 ms depolarizing step from -70 mV to +40 mV, followed by hyperpolarization to -20 mV for 500 ms. Currents were measured at the steady state of +40mV voltage step.

No corrections for liquid junction potentials (estimated as -20 mV by comparing the KCNQ2 reversal potential with the calculated Nernst potential for potassium) were applied. The current signal was sampled at 0.625 kHz.

Statistics

The raw data of thallium flux assay were analyzed and exported from manufacturer's software package provided by Hamamatsu Photonics (Hamamatsu, Japan). Automated patch clamp data were analyzed in IonWorks 2.0.4.4 (Moledular Devices Corp., Sunnyvale, CA) and then exported for further analysis by Excel (Microsoft, CA) and Origin 6.

Table 7. Ricerca Profiling of SID ML213.

SID/VU#	Structure	Primary Biochemical Assay	Species	% inhibition @ 10 μM
ML213		*No Significant Activity Found*		